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A NOVEL GENE INVOLVED IN BRASSINOSTEROID RESPONSES

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BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION:

The present invention relates to a novel gene. In particular, the present invention relates to a novel gene in plants which encodes a protein having the function of controlling an in-vivo signal transduction system in a physiological reaction system against brassinosteroid hormone.

2. DESCRIPTION OF THE RELATED ART:

Transposons are mutagenic genes which are known to be ubiquitous in animal, yeast, bacterial, and plant genomes. Transposons are classified into two classes, Class I and Class II, depending on their transposition mechanisms. Transposons belonging to Class II are transposed in the form of DNAs without being replicated. Known Class II transposons include the Ac/Ds, Spm/dSpm and Mu elements of Zea mays (Fedoroff, 1989, Cell 56, 181-191; Fedoroff et al., 1983, Cell 35, 235-242; Schiefelbein et al., 1985, Proc. Natl. Acad. Sci. USA 82, 4783-4787), and the Tam element of Antirrhinum majus (Bonas et al., 1984, EMBO J., 3, 1015-1019). Class II transposons are widely used for gene isolation techniques which utilize transposon tagging. Such techniques utilize the fact that a transposon induces physiological and morphological changes when inserted into genes. The affected gene can be isolated by detecting such changes (Bancroft et al., 1993, The Plant Cell, 5, 631-638; Colasanti et al., 1998, Cell, 93, 593-603; Gray et al., 1997, Cell, 89, 25-31; Keddie et al., 1998, The Plant Cell, 10, 877-887; Whitham et al., 1994, Cell, 78, 1101-1115).

Transposons belonging to Class I, also referred to as retrotransposons, are replicated and transposed via RNA intermediates. Class I transposons were first identified and characterized in Drosophila and in yeasts. However, recent studies have revealed that Class I transposons are ubiquitous in plant genomes and account for a substantial portion of the genomes (Bennetzen, 1996, Trends Microbiolo., 4, 347-353; Voytas, 1996, Science, 274, 737-738). A large majority of retrotransposons appear to be inactive. Recent studies indicate that some of these retrotransposons are activated under stress conditions such as injuries, pathogenic attacks, or cell culture (Grandbastien, 1998, Trends in Plant Science, 3, 181-187; Wessler, 1996, Curr. Biol. 6, 959-961; Wessler et al., 1995, Curr. Opin. Genet. Devel. 5, 814-821). Activation under stress conditions has been reported for Tnt1A and Tto1 in tobacco (Pouteau et al., 1994, Plant J., 5, 535-542; Takeda et al., 1988, Plant Mol. Biol., 36, 365-376), and Tos17 in rice (Hirochika et al., 1996, Proc. Natl. Acad. Sci. USA, 93, 7783-7788), for example.

The Tos17 retrotransposon of rice is one of the most-extensively studied plant Class I elements in plants. Tos17 was cloned by an RT-PCR method using a degenerate primer prepared based on a conservative amino acid sequence in reverse transcription enzyme domains between Ty1-copia retroelements (Hirochika et al., 1992, Mol. Gen. Genet., 233, 209-216). Tos17 is 4.3kb long, and has two 138bp LTRs (long chain terminal repetitions) and PBS (primer binding sites) complementary to the 3' end of the start methionine tRNA (Hirochika et al., 1996, supra). Tos17 transcription is strongly activated through tissue culture, and its copy

number increases with culture time. In Nipponbare, a model Japonica cultivar used for genome analysis, two copies of Tos17 are initially present, which are increased to 5 to 30 copies in a regenerated plant after tissue culture (Hirochika et al., 1996, supra). Unlike Class II transposons which were characterized in yeasts and Drosophila, Tos17 is transposed in chromosomes in random manners and causes stable mutation, and therefore provides a powerful tool for functional analysis of rice genes (Hirochika, 1997, Plant Mol. Biol. 35, 231-240; 1999, Molecular Biology of Rice (ed. by K. Shimamoto, Springer-Verlag, 43-58).

SUMMARY OF THE INVENTION

The present invention relates to a polynucleotide encoding a plant gene capable of controlling a signal transduction system for brassinosteroid hormone, the polynucleotide encoding an amino acid sequence from Met at position 1 to Arg at position 1057 of SEQ ID NO: 2 in the SEQUENCE LISTING, including any polynucleotide encoding an amino acid sequence in which one or more amino acids are deleted, substituted or added to the amino acid sequence.

In one embodiment of the invention, the polynucleotide may be derived from rice.

In another embodiment of the invention, the polynucleotide may be as represented by SEQ ID NO: 1 in the SEQUENCE LISTING.

The present invention further relates to methods for controlling various effects in plants in which

brassinosteroid hormone is involved, e.g., growth promotion, yield increase, quality improvement, maturation enhancement, and tolerance against biotic and abiotic stresses.

The inventors diligently conducted systematic analyses of phenotypes of plants having a newly transposed To17 copy and sequences adjoining Tos17 target sites with respect to rice. As a result, the inventors found a dwarf rice mutation obtained from Tos17 insertion, and isolated the gene responsible for this mutation by utilizing Tos17 as a tag, thereby accomplishing the present invention.

Thus, the invention described herein makes possible the advantage of providing a novel plant gene which can be provided by using Tos17.

This and other advantages of the present invention will become apparent to those skilled in the art upon reading and understanding the following detailed description with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures **1A** and **1B** are photographs showing a brassinosteroid non-sensitive mutant having Tos17 inserted therein, which was found among regenerated Akitakomachi lineage. On the left of each figure is shown a brassinosteroid non-sensitive mutant having Tos17 inserted therein. On the right of each figure is shown a wild type plant body. Figure **1A** evidences an influence toward dwarfism and upright form. Figure **1B** evidences an influence toward malformation of grain hulls.

Figures **2A** and **2B** are photographs showing a brassinosteroid non-sensitive mutant having Tos17 inserted therein, which was found among regenerated Nipponbare lineage. On the left of each figure is shown a brassinosteroid non-sensitive mutant having Tos17 inserted therein. On the right of each figure is shown a wild type plant body. Figure **2A** evidences an influence toward dwarfism and upright form. Figure **2B** evidences an influence toward malformation of grain hulls.

Figure **3A** shows a Northern analysis autoradiogram of RNA extracted from the leaves of a brassinosteroid non-sensitive mutant (Akitakomachi) and RNA extracted from various organs of a wild type rice plant (Nipponbare).

Figure **3B** shows a Northern analysis autoradiogram of RNA extracted from brassinosteroid non-sensitive mutants and RNA extracted from wild type rice plants. The left-hand side of Figure **3B** shows a comparison between wild types and mutants obtained by using a 5' probe. The right-hand side of Figure **3B** shows a comparison between wild types and mutants obtained by using a 3' probe.

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Figure **4** shows an amino acid sequence of the novel rice gene which controls a physiological reaction system induced by brassinosteroid hormone, together with characteristic sequences found therein (where nuclear localization signals and an ATP/GTP binding motif can be observed).

Figure **5A** shows a brassinosteroid leaf blade bending experimentation using a mutated line (A0369) derived

from Akitakomachi. The left-hand side shows results of wild type plants, whereas the right-hand side shows results of mutants.

Figure **5B** shows a brassinosteroid leaf blade bending experimentation using a mutated line (NC6148) derived from Nipponbare. The left-hand side shows results of wild type plants, whereas the right-hand side shows results of mutants.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a method for producing an improved plant, the method utilizing a novel plant gene which can be provided by using Tos17.

According to the present invention, there is provided a polynucleotide encoding a plant gene capable of controlling various effects in which brassinosteroid hormone is involved. As used herein, the term "capable of controlling various effects" means the ability to control various effects in plants in which brassinosteroid hormone is involved, e.g., growth promotion, yield increase, quality improvement, maturation enhancement, and tolerance against biotic and abiotic stresses, including dwarfism, upright form, and malformation of grain hulls, thereby providing a number of agriculturally useful effects as are attained by treatments with brassinosteroid hormone agricultural chemicals. The term "plants" encompasses both monocotyledons and dicotyledons.

A polynucleotide encoding a plant gene capable of controlling a signal transduction system for

brassinosteroid hormone according to the present invention is, for example, a polynucleotide encoding an amino acid sequence from Met at position 1 to Arg at position 1057 of SEQ ID NO: 2 in the SEQUENCE LISTING, including any polynucleotide encoding an amino acid sequence in which one or more amino acids are deleted, substituted or added to the aforementioned amino acid sequence.

A polynucleotide encoding a plant gene capable of controlling various effects in which brassinosteroid hormone is involved encompasses any polynucleotides which have at least about 80% sequence homology, preferably at least about 85% sequence homology, and more preferably at least about 90% sequence homology, still more preferably at least about 95% sequence homology, and most preferably at least about 99% sequence homology, with an amino acid sequence from Met at position 1 to Arg at position 1057 of SEQ ID NO: 2 in the SEQUENCE LISTING, so long as they are capable of controlling various effects in plants in which brassinosteroid hormone is involved. The term "sequence homology" indicates a degree of identity between two polynucleotide sequences to be compared with each other. The rate (%) of sequence homology between two polynucleotide sequences for comparison is calculated by, after optimally aligning the two polynucleotide sequences for comparison, obtaining a matched position number indicating the number of positions at which identical ("matched") nucleic acid bases (e.g., A, T, C, G, U, or I) are present in both sequences, dividing the matched position number by total number of bases in the polynucleotide sequences for comparison, and multiplying the quotient by 100. The sequence homology can be calculated by using the following sequencing tools, for example: a Unix base program designated GCG Wisconsin

Package (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive Madison, Wisconsin, USA 53711; Rice, P. (1996) Program Manual for EGCG Package, Peter Rice, The Sanger Centre, Hinxton Hall, Cambridge, CB10 1RQ, England), and the ExPASy World Wide Web molecular biology server (Geneva University Hospital and University of Geneva, Geneva, Switzerland).

Cells into which genes have been introduced are first selected based on drug resistance, e.g., hygromycin resistance, and then regenerated into plant bodies by using usual methods.

The terminology and laboratory procedures described throughout the present specification are directed to those which are well-known and commonly employed in the art. Standard techniques may be used for recombination methods, polynucleotide synthesis, microorganisms culturing, and transformation (e.g., electroporation). Such techniques and procedures are generally known from various standard textbooks available in the field or by way of the present specification (including a generally-referenced textbook by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Such literature is incorporated herein by reference.

The polynucleotide according to the present invention can be obtained by using the method described herein, for example. However, the polynucleotide according to the present invention may also be obtained by any chemical synthesis process based on the sequence disclosed herein. For example, the polynucleotide according to the present

invention may be synthesized by using a polynucleotide synthesizer available from Applied Bio Systems in accordance with the instructions provided by the manufacturer.

Methods of PCR amplification are well-known in the art (PCR Technology: Principles and Applications for DNA Amplification, ed. HA Erlich, Freeman Press, NewYork, NY (1992); PCR Protocols: A Guide to Methods and Applications, Innis, Gelfland, Snisky, and White, Academic Press, San Diego, CA(1990); Mattila et al. (1991) Nucleic Acids Res. 19: 4967; Eckert, K.A. and Kunkel, T. A. (1991) PCR Methods and Applications 1: 17; PCR, McPherson, Quirkes, and Taylor, IRL Press, Oxford). Such literature is incorporated herein by reference.

(Examples)

Hereinafter, the present invention will be described by way of examples which are of illustrative but non-limiting nature.

(Example 1: Activation of Tos17 via culture)

Using fully ripened seeds of Nipponbare and Akitakomachi, which are varieties of Japonica subspecies, induction of calli and cell suspension culture were carried out as described earlier (Hirochika et al., 1996, supra). The activation of Tos17 which was used for gene destruction was carried out following the method of Ohtsuki (1990) (rice protoplast culture system, Food and Agricultural Research Development Association). In summary, fully ripened seeds of rice were cultured in an MS medium having 2,4-dichlorophenoxyacetic acid (2,4-D) added thereto (2 mg/ml) (Ohtsuki (1990), supra) (25℃, 1 month), to induce callus formation. The resultant calluses were cultured for

5 months in an N6 liquid medium having 2,4-D added thereto (Ohtsuki (1990), supra), and thereafter placed on a redifferentiation medium (Ohtsuki (1990), supra), whereby redifferentiated rice plants were obtained (first generation (R1) plants).

(Example 2: Isolation of sequences adjoining Tos17)

Utilizing each of the regenerated R1 rice plants obtained according to Example 1 as a first strain, about 1000 R1 seeds were collected from each strain and grown on a paddy field to obtain second generation (R2) plants, which were subjected to a morphological analysis. As a result of observing the phenotypes of the respective plant bodies in the R2 group, it was learned that about 1/4 of the R2 group of an Akitakomachi strain A0369 exhibit the "dwarfism, upright form, and malformation of grain hulls" phenotype (Figures **1A** and **1B**). In the regenerated group of Akitakomachi, dwarfism, upright form, and malformation of grain hulls were observed for brassinosteroid insensitive mutants (Figure **1A**, left, and Figure **1B**, left), as compared with the wild type (Figure **1A**, right, and Figure **1B**, right). The isolation of adjacent sequences of transposed Tos17, which is co-segragating with the phenotypes, was carried out by an IPCR method (Ochman et al., Genetics Nov; 120(3): 621-3(1988) and Triglia et al., Nucleic Acids Res Aug 25; (16): 8186(1988)). The total DNA of A0369 was digested with XbaI, and a ligation process was performed in a large quantity of solution, thereby obtaining self-ligated circular molecules. In the self-ligated circular molecules, the adjacent sequences are flanking the internal sequence of Tos17. As a result, amplification was successfully carried out by usual PCR methods using an outward primer pair (T17TAIL3: GAGAGCATCATCGGTTACATCTTCTC; T17-1950R:

TCTAGCAGTCTCAATGATGTGGCG) based on the known sequence of Tos17.

(Example 3: Search for alleles)

Using the sequence obtained according to Example 2, lineage in which Tos17 had been inserted at a different site in the same gene was selected by PCR from the regenerated rice group of Nipponbare. As a result, a line (NC6148) which similarly exhibited dwarfism, upright form, and malformation of grain hulls were observed for brassinosteroid was selected. That is, in the regenerated rice group of Nipponbare, as well, dwarfism, upright form, and malformation of grain hulls were observed for brassinosteroid insensitive mutants (Figure **2A**, left, and Figure **2B**, left), relative to the wild type (Figure **2A**, right, and Figure **2B**, right). It was concluded that these common mutations were results of the same gene having been destroyed.

(Example 4: Expression and analysis of the causative gene)

From the group of R2 rice plants(selfed progeny from the A0369 and NC6148 strains) obtained according to Examples 2 and 3, individuals exhibiting mutation were identified from normal individuals. RNA was prepared from both groups of individuals, and the expression specificity was analyzed through Northern analysis. After agarose electrophoresis, the RNA obtained from individuals exhibiting mutation and the RNA obtained from normal individuals were allowed to adsorb to nylon membranes. DNA fragments which were obtained by amplifying via PCR a sequence (positions 5775-6638 of the genomic sequence) on the 5' side and a sequence (positions 8175-8765 of the genomic sequence) on the 3' side of the Tos17 insertion site

in both mutated lines were labeled with ^{32}P-dCTP. By using these as probes, expression specificity was analyzed through Northern analysis (Figures **3A** and **3B**). As seen from the Northern analysis autoradiogram shown in Figure **3A**, a band (about 4.3 kb) indicated by an arrow was confirmed to be expressed in all observed organs of the wild type. However, in the mutants, transcripts of abnormal sizes were observed due to the insertion of Tos17, indicating that the natural function of the wild type is lost (Figure **3B**).

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(Example 5: Structural analysis of the causative gene)

Using the sequence obtained according to Example 2 as a probe, the corresponding cDNA and genomic clone were obtained from a cDNA library and a genomic library. Their structures are shown in SEQ ID Nos: 1 and 3. It was learned that this gene includes 6 exons and 5 introns, encoding 1057 amino acids, and that Tos17 had been inserted at the 4th and 5th exons in two mutants, respectively. Moreover, motif search results suggested the presence of nuclear localization signal 1 (amino acid residues 329-367 of SEQ ID NO: 2, Robbins & Dingwall consensus sequence; a search result by PSORT program (http://psort.ims.u-tokyo.ac.jp/)) and nuclear localization signal 2 (amino acid residues 457-460, 595-600 of SEQ ID NO: 2, 4 amino acid nuclear localization pattern signal; a search result by PSORT program (http://psort.ims.u-tokyo.ac.jp/)) as well as the presence of an ATP/GTP binding domain (amino acid residues 526-533 of SEQ ID NO: 2; a search result by a motif search service on Genomenet(http://www.genome.ad.jp/)). Thus, the possibility of this gene being involved in signal transduction was suggested (Figure **4**).

(Example 6: brassinosteroid sensitivity evaluation)

The present gene was deduced to be a factor involved in the signal transduction system for plant hormones, taking note of the facts that the present gene was expressed in all plant bodies and that pleiotropic influences resulted from destroying this gene, as well as the possibility that the gene might be a factor involved in the signal transduction system. Presuming that the signal transduction system is that for brassinosteroid hormone in view of the resultant upright form, the inventors performed a leaf blade bend response test as a brassinosteroid response test, by using brassinolide, which is one kind of brassinosteroid hormone. The second leaf of rice which was allowed to germinate in the dark was cut off, and immersed in a 1 ng/ml of brassinosteroid solution for 48 hours. The wild type individuals having the wild type genes showed bending of the leaf blades and leaf sheath junctions (left-hand side in Figures **5A** and **5B**), showing response to brassinolide, whereas mutant individuals showed little bending thereof (right-hand side in Figures **5A** and **5B**), indicating that the destruction of the present gene resulted in the loss of response to brassinosteroid. From the above results, it was revealed that the present gene is a gene involved in the signal transduction system for brassinosteroid hormone.

The above examples are illustrative, and by no means limiting, of various aspects of the present invention and the manners in which the oligonucleotide according to the present invention can be made and utilized.

Thus, according to the present invention, a novel polynucleotide is provided which is capable of controlling various effects in which brassinosteroid hormone is involved,

the polynucleotide being of use in plant breeding. By introducing the present polynucleotide into plants and artificially controlling various effects in which brassinosteroid hormone is involved, it is expected that effects such as growth promotion, yield increase, quality improvement, maturation enhancement, and tolerance against biotic and abiotic stresses can be controlled, thereby providing a number of agriculturally useful effects as are attained by treatments with brassinosteroid hormone agricultural chemicals.

Various other modifications will be apparent to and can be readily made by those skilled in the art without departing from the scope and spirit of this invention. Accordingly, it is not intended that the scope of the claims appended hereto be limited to the description as set forth herein, but rather that the claims be broadly construed.